from a depletion of succinic semialdehyde. The role of this aldehyde in the brain may be as important as GABA and investigation of its function may prove fruitful.

*Acknowledgement*—The assistance of Mr. N. J. Crittenden is gratefully acknowledged.

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## 5-Bromo-2′-deoxycytidine (BCDR)–II. Studies with murine neoplastic cells in culture and *in vitro*

(*Received by Editors* 29 *July* 1961; *Received by Publishers* 1 *August* 1961)

IN CONJUNCTION with studies of the stability of 5-bromo-2′-deoxycytidine (BCDR) to metabolic degradative attack *in vivo*,[1] the presently reported work was undertaken to investigate the toxicity, metabolism and mechanism of action of BCDR with mammalian neoplastic cells, both in culture and under conditions of incubation *in vitro*.

*Effect of BCDR on murine neoplastic mast cells grown in culture.* The culture medium,[2] cell line (murine neoplastic mast cells, P815Y),[3] and techniques[4] have been described previously, with the exception that the Coulter Cell Counter was used for determinations of the number of cells.

BCDR in graded concentrations, 1–400 $\mu$M ,in the medium progressively inhibited the growth of the cells, although complete inhibition did not occur even with 400 $\mu$M. With that level, cell reproduction was limited to only two divisions; while with the same level of 5-iodo-2′-deoxyuridine (IUDR) or 5-bromo-2′-deoxyuridine (BUDR), growth was limited to but a single division. Following exposure of the cells to BCDR for three cell-generations and subsequent transfer to drug-free medium, a progressive decrease in cell viability occurred with increasing concentration of the agent.

Inhibition of growth of BCDR could be prevented partially by addition to the medium of either thymidine (TDR) or 2′-deoxycytidine (CDR); however, much higher levels of CDR than of TDR were required for comparable degrees of prevention of growth inhibition, a finding which supports the concept that BCDR and TDR are handled analogously by the cells. However, complete reversal by TDR could not be obtained, since levels greater than about 60 $\mu$M are toxic to the cells, a phenomenon which has been described previously and explained.[5]

Both BUDR and BCDR partially prevented the inhibition of cell growth caused by otherwise totally inhibitory levels of 5-fluoro-2′-deoxyuridine (FUDR), but neither compound was as effective as TDR.

The uptake and metabolism of $^3$H-BUDR and $^3$H-BCDR *in vitro* were compared using suspensions of cells, $5 \times 10^7$ per ml, incubated with tracer quantities of nucleosides of high specific activity. After periods of incubation of 5, 15 and 30 min, respectively, the tubes were chilled, carrier BUDR and BCDR were added, and the cells were sedimented by centrifugation. The surface of the packed cells and the walls of the tube were washed once with fresh medium to remove most of the residual compound; the cell-pack was then extracted three times with cold 5% trichloroacetic acid (TCA), followed by extraction with 5% TCA at 90°C for 30 min. After the TCA was removed from the extracts with ether and water was removed *in vacuo*, the residues derived from the extractions with cold TCA were chromatographed on Whatman no. 1 filter paper in butanol:water (344:56, v/v). No significant

radioactivity on the chromatogram was associated with the carrier BUDR derived from the cold acid soluble extracts of the cells incubated with $^3$H-BCDR. However, these extracts contained a phosphorylated derivative of BUDR which had the same $R_f$ on Whatman no. 1 filter paper, developed in isopropanol:HCl:water (260:64:76, v/v), as that of authentic deoxyuridine 5′-monophosphate; much higher levels of the same phosphorylated derivative were found in the cold acid-soluble extracts of the cells incubated with $^3$H-BUDR. The hot TCA-soluble extracts of the cold acid-insoluble residues also contained radioativity which resulted from incorporation of the radioactive precursors into the cellular DNA; moreover, much more radioactivity was present in the extracts derived from cells incubated with $^3$H-BUDR than was present in those derived from cells incubated in the presence of $^3$H-BCDR. This finding suggests that BCDR was converted to phosphorylated derivatives of BUDR, with subsequent incorporation into the DNA of the cell, and that this occurred at a more rapid rate with BUDR than with BCDR. In comparison with the results obtained with $^3$H-BUDR, those with $^3$H-BCDR might be attributed to either reduced transport of $^3$H-BCDR through the cell wall or to reduced conversion of BCDR to BUDR-phosphates *via* deamination and phosphorylation, or to both circumstances.

Enzymically hydrolysed DNA prepared from cells incubated with $^3$H-BCDR contained essentially all its radioactivity as 5-bromo-2′-deoxyuridine 5′-monophosphate (Table 1); this fraction replaced an equivalent amount of thymidylic acid (dTMP) (46 per cent of that of the newly synthesized DNA); the sum of these nucleotides, 2·24 μmoles, corresponded almost exactly to the amount of 2′-deoxyadenylic acid (dAMP) isolated, 2·14 μmoles, in accordance with the Watson-Crick[7] formulation for base-pairing.

TABLE 1. INCORPORATION OF BCDR AS 5-BROMO-2′-DEOXYURIDYLIC ACID INTO THE DNA OF MURINE NEOPLASTIC MAST CELLS (P815Y) IN CULTURE

| Deoxyribonucleotide | μMoles of nucleotide in DNA | Per cent replacement of thymidylic acid by BUDR-5′-P |
|---|---|---|
| dAMP | 2·14 | — |
| dTMP | 1·40 | 46·4 |
| BUDR-5′-P | 0·84 | 46·4 |
| | 2·24 | |

Murine neoplastic mast cells (P815Y) were grown in mass culture for slightly more than two cell divisions in the presence of $2 \times 10^{-5}$ M $^3$H-BCDR (1·45 μc/μmole). The washed harvested cells (0·9 g) were homogenzied in 0·25 M sucrose–0·002 M $CaCl_2$ and the nuclei were sedimented by centrifugation at 800 **g** for 15 min, washed once with fresh sucrose–$CaCl_2$, and the supernatant fractions discarded. The nuclei were dissolved in cold 1·7 M NaCl which was then added to 2 vols of 95% ethanol to precipitate the nucleoprotein. The precipitate was redissolved in 1·7 M NaCl and the solution was homogenized with an equal volume of chloroform:octyl alcohol (5:1) three times, in order to remove the protein. The DNA was again precipitated by the addition of the NaCl solution to ethanol and the dried preparation was degraded enzymically with pancreatic deoxyribonuclease (Worthington), then with a purified snake venom phosphodiesterase which had no significant deaminase activity. The resulting mixture of deoxyribonucleotides was separated on Dowex-1 formate columns (1 × 25 cm) by gradient elution with a solution of ammonium formate (1 M, pH 4·1).[6] The quantity of each deoxyribonucleotide recovered was determined spectrophotometrically on the pooled samples; the concentration of BUDR-5′-P was calculated on the basis of the specific activity of the initial $^3$H-BCDR and the compound was further characterized as BUDR by paper chromatography in butanol:water, following dephosphorylation of the nucleotide with alkaline phosphatase.

The pathway by which BCDR is converted to phosphorylated derivatives of BUDR in the P815Y cell can be deduced from data concerned with cross-resistance to BCDR (and other related compounds) of a cloned sub-line (P815Y/FUDR) selected for resistance to FUDR.[5] A much higher level (about 1000-fold higher) of FUDR (50 per cent growth inhibitory concentration: $2·6 \times 10^{-6}$ M) was required to inhibit the growth of these cells than was needed for the inhibition of the growth of the parent cells. The P815Y/FUDR cells also were cross-resistant to levels of either TDR, IUDR or BUDR which were profoundly toxic for the sensitive parent strain; however, the cells were *not* resistant to 5-fluoro-2′-deoxycytidine (FCDR). Resistance to the first three cited compounds has been shown to

result from a greatly reduced level of thymidine kinase activity in the P815Y/FUDR cell.[5] Lack of cross-resistance to FCDR implies that the metabolic block which results from deletion or suppression of thymidine kinase activity may be circumvented by direct phosphorylation and deamination of FCDR, with intracellular formation of the 5′-monophosphate of FUDR, the active inhibitor of thymidylic acid synthetase.[8, 9] Cross-resistance to BCDR infers that, in the P815Y/FUDR cells, activation of this compound does *not* proceed to a significant degree by the same pathway as does FCDR. *A priori*, its conversion to phosphorylated derivatives of BUDR in the parent line may occur following deamination. Assay of enzyme preparations of the parent cells for the presence or absence of significant activity for the deamination of BCDR to BUDR, prior to phosphorylation, will be necessary to confirm this hypothesis.

*Deaminase activity* in vitro *of a cell-free preparation of murine L5178Y lymphoblasts.* Cells were harvested 6 days following the inoculation of AKR × DBA/2 $F_1$-hybrid mice with L5178Y ascites cells; after centrifugation the cells were washed once with saline. Packed cells (3 ml), Tris buffer (0·05 M, pH 7·9) and glass beads (200 micron, 7 ml) were shaken for 20 sec in a Nossal vibrator. Following filtration through glass wool, the filtrate was centrifuged at 10,000g for 10 min. The reaction mixture (1·2 ml) contained $MgCl_2$ (5·5 μmoles), ATP (110 μmoles), Tris buffer (110 μmoles, pH 7·9), $^3$H-BCDR (0·1 μmole, $6 \times 10^4$ cpm), and cell-free extract (0·5 ml). The reaction was terminated, after incubation for 90 min at 37 °C, by the addition of TCA. Both the composition of the reaction mixture and the conditions used were similar to those described by Weissman *et al.*[10]

The acid-soluble fraction was separated into two radioactive areas by paper chromatography in the ethylacetate:phosphate buffer system.[11] One area, which contained about 9 per cent of the total radioactivity, was identified as BUDR. The other area, which was closely associated with the point of origin, was eluted and rechromatographed in the butanol:ammonia system;[12] essentially all radioactivity of this latter area migrated with authentic BCDR. No radioactivity was observed at the point of origin, in which nucleotides, if formed, would have been present. Under these conditions of incubation, the formation of thymidylic acid from $^3$H-TDR has been demonstrated previously.[13] Thus, the cell-preparations contained enzymic activity for the deamination of BCDR, but the data do not establish whether this deamination occurred before or after phosphorylation; it is conceivable that the extracts may have rapidly hydrolyzed any phosphorylated derivatives of either BCDR or BUDR, or both.

*Effect of BCDR on the utilization of TDR for the biosynthesis of DNA by various neoplasms* in vitro. Although studies of neoplastic mast cells in culture (see earlier section) demonstrated the extensive incorporation into DNA of 5-bromo-2′-deoxyuridylic acid, derived from BCDR, another possible site of action of this agent could involve an inhibition, at either nucleoside or nucleotide levels, of the utilization of TDR for the biosynthesis of DNA-thymine. Evidence for this type of inhibition has been provided in earlier studies in which the capacity of either orotic acid or thymidine to serve as a precursor of DNA-thymine was markedly reduced by BUDR and IUDR[14, 15]; of these compounds, BUDR was the more active. The decreased incorporation of TDR in the presence of IUDR reflects an inhibition of the itilization of either TDR, thymidylic acid or the triphosphate of TDR, presumably by IUDR or the corresponding phosphorylated derivative.[13]

Since BCDR also inhibits the incorporation of $^3$H-TDR into the DNA-thymine of intact Ehrlich ascites carcinoma cells and L5178Y lymphoblasts (Table 2), the question arises as to whether BCDR or its phosphorylated derivatives possess antimetabolic activity *prior* to deamination. Evidence for deaminase activity in cell-free extracts of the L5178Y cells has been provided; accordingly, the extent of inhibition observed in Table 2 could be accounted for by a deamination of the added BCDR to the extent of only 10–20 per cent.

It appears that BUDR is more potent than BCDR as an inhibitor of the utilization of TDR for the formation of DNA-thymine. Thus, BUDR, at a ratio of 20:1, inhibited by 96 per cent the utilization of TDR for the biosynthesis of DNA-thymine by murine Ehrlich ascites carcinoma cells *in vitro*,[14] whereas BCDR, even at a ratio of 50:1, exerted only 53 per cent inhibition (Table 2).

## DISCUSSION

The results indicate that with three types of murine neoplastic cells (mast cells, P815Y; lymphoblasts, L5178Y; and Ehrlich ascites carcinoma cells), BCDR is deaminated; incorporation into DNA, in the form of BUDR-5′-P in place of thymidylic acid, has been demonstrated with the mast cells.

Although not unequivocally established that BCDR can be deaminated *directly* (i.e., without prior phosphorylation), the studies with mast cells resistant to BCDR (but not to FCDR), strongly indicate that primary deamination of BCDR does occur with these cells. In other cases, however, the formation of BCDR-5′-P, with deamination by deoxycytidylate deaminase, to form BUDR-5′-P, also may occur. Thus, a possibility remains that this latter pathway could predominate in some types

TABLE 2. EFFECT OF BCDR ON THE UTILIZATION OF $^3$H-THYMIDINE FOR THE BIOSYNTHESIS OF DNA-THYMINE BY TWO MURINE NEOPLASMS *in vitro**

| Cells | Amount of BCDR in μmoles | Relative specific activity of isolated DNA-thymine† |
|---|---|---|
| Ehrlich ascites | None | 1·00 |
| Carcinoma cells | 0·5 | 0·66 |
| | 5·0 | 0·47 |
| L5178Y | None | 1·00 |
| Lymphoblasts | 0·5 | 1·06 |
| | 5·0 | 0·65 |

* The reaction mixtures (final volume, 2·7 ml) contained packed cells (0·25 ml), horse serum (0·2 ml), $^3$H-TDR (0·1 μmole, 5 μc), in the absence or presence of BCDR in the amounts listed in the table, and Totter's modification of Chamber's solution.[16] The incubations were conducted in duplicate in 20-ml beakers in a Dubnoff metabolic shaker at 37°C (air); agitation was at 90 cycles per min for 4 hr.

† The specific activity of the control DNA-thymine was equated to 1·00.

of neoplastic cells, and this is deserving of further investigation, despite the finding that, in mice,[1] BCDR is less resistant to metabolic degradation than various studies *in vitro* had suggetsed. Since 5-bromocytosine does not appear to be formed from BCDR, at least in mouse liver *in vitro*,[1] dehalogenation to free uracil presumably reflects the intermediation of 5-bromouracil derived from deaminated BCDR, despite earlier indications that BCDR did not participate in reactions leading to the removal of the deoxyribose-moiety.[17] Accordingly, the problems for the immediate future involve direct determinations of the relative rates and mechanisms of anabolism and catabolism of BUDR and BCDR, particularly in man. Possibly of even greater potential chemotherapeutic interest will be comparable studies with IUDR and 5-iodo-2′- deoxycytidine.[18]

*Acknowledgements*—These studies were supported by grants (CY-2817 and C-5262) from the National Cancer Institute and by grants (T17 and T23) from the American Cancer Society. We are grateful to Dr. P. K. Chang for the preparation of the 5-bromo-2′-deoxycytidine and to the Cancer Chemotherapy National Service Center, U.S. Public Health Service, for a supply of 5-bromo-2′-deoxyruidine. Our thanks are due to Dr. G. A. Fischer and Dr. N. R. Morris of this department for their assistance in determining cross-resistance to TDR and FCDR in the P815Y/FUDR cells.

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## Influence of nicotinic acid and nicotinamide on diphosphopyridine nucleotide (DPN) synthesis in ascites tumor cells*

(*Received by Editors* 17 *July* 1961; *Received by Publishers* 1 *August* 1961)

IN THE biosynthesis of DPN from nicotinic acid (NA) and nicotinamide (NAM), desamido-DPN was identified as the common precursor in human erythrocytes and mouse liver tissue.[1, 2] Comparing the relative abilities of both types of cells to synthesize DPN from the respective vitamins, however, it is apparent that erythrocytes depend almost totally on NA and cannot utilize NAM to any appreciable extent, while liver tissue shows a preference for NAM as a DPN-precursor.

Roitt[3] demonstrated that alkylating agents of the ethyleneimine type inhibit glycolysis in tumor cells by interference with the synthesis of DPN. The decrease in the intra-cellular concentration of DPN associated with the action of the alkylating agent could be prevented when NAM was added to the incubation medium;[4] NA was ineffective in this respect. Concluding from these experiments that tumor cells may depend on NAM rather than NA for DPN-synthesis, it was desirable to investigate the specific utilization of these vitamins by a tumor and to compare the results with the biochemical transformations observed in a non-neoplastic tissue of the same animal.

From a group of 12 white Swiss–Webster mice (weight 50–60 g) with Ehrlich ascites tumor, 4 animals were injected intramuscularly with NA, 170 mg/kg, 4 received NAM in equal doses, and the remainder served as controls. Ten hours after the injections, concentrations of oxidized DPN in liver and tumor homogenates were estimated spectrophotometrically, using crystalline yeast alcohol dehydrogenase according to the method of Holzer.[5]

The results (see Table 1) indicate that the capacity of the tumor cells for DPN-synthesis from either NA or NAM is only about one-tenth that of liver tissue of the same animal. Comparing the specific utilization of the precursors for the synthesis of DPN, NAM was approximately four times as effective as NA in both liver and tumor, suggesting a similar biochemical mechanism for DPN-synthesis in these tissues. The limited response of the tumor cell is not related to insufficient transfer of the precursors into the ascitic fluid. No significant different in DPN levels was observed when NAM and NA were injected intraperitoneally instead of intramuscularly (unpublished data). The possibility that the tumor cell has a poor capacity to incorporate the precursors can be disregarded, since Holzer and Boltze[6] using $^{14}$C-labeled NAM and NA showed that both vitamins penetrate freely into ascites tumor cells. Further investigations are needed to determine whether the same intermediates and enzymic sequences occur in this tumor.

* This investigation was supported by grants (CY-4849 and RG-6956) from the National Institutes of Health, U.S. Public Health Service.

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